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<p>(54) Title: SESQUITERPENE LACTONES SPECIFICALLY INHIBIT ACTIVATION OF NF-κB BY PREVENTING THE DEGRADATION OF IκB-α AND IκB-β</p> <p>(57) Abstract</p> <p>Extracts from certain Mexican indian medicinal plants used in traditional indigenous medicine for the treatment of inflammations contain sesquiterpene lactones (SLs), which specifically inhibit the transcription factor NF-κB (1). Here we show that SLs prevented the activation of NF-κB by different stimuli such as phorbol esters, tumor necrosis factor (TNF)-α, ligation of the T-cell receptor and hydrogen peroxide in various cell types. Treatment of cells with SLs prevented the induced degradation of IκB-α and IκB-β by all these stimuli, suggesting that they interfere with a rather common step in the activation of NF-κB. SLs did neither interfere with DNA-binding activity of a ctivated NF-κB nor with the activity of the protein tyrosine kinases p59^{lyn} and p60^{src}. Micromolar amounts of SLs prevented the induced expression of the NF-κB target gene ICAM-1. Inhibition of NF-κB by SLs resulted in an enhanced cell-killing of murine fibroblast cells by TNF-α. SLs lacking an exomethylene group in conjugation with the lactone function displayed no inhibitory activity on NF-κB. The analysis of the cellular redox-state by FACS showed that the SLs had no direct or indirect anti-oxidant properties.</p>		

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Sesquiterpene lactones specifically inhibit activation of NF- κ B by preventing the degradation of I κ B- α and I κ B- β

Field of the invention

The present invention is concerned with the use of extracts from certain Mexican indian medicinal plants applied for the treatment of inflammations. These extracts contain sesquiterpene lactones (SLs), which specifically inhibit the transcription factor NF- κ B.

Background of the invention

The transcription factor NF- κ B is one of the key regulators of genes involved in the immune and inflammatory response (for review see 2). In mammalian cells, NF- κ B is composed of a homo- or heterodimer of various DNA-binding subunits. Five different DNA-binding subunits share a N-terminal homology domain, which confers DNA-binding, dimerization, nuclear translocation and interaction with the inhibitory I κ B proteins (for review see 3, 4). In most cell types these proteins sequester NF- κ B, which is frequently a heterodimer of the p50 and p65 (RelA) subunits, in the cytoplasm by masking their nuclear localization sequence. Constitutive NF- κ B activity in the cell nucleus can only be detected in certain neurons, some cells of the monocyte/macrophage lineage and B cells (for review see 5, 6). Stimulation of cells with a variety of pathogenic agents including inflammatory cytokines, phorbol esters, UV irradiation and oxidants finally leads to the intracellular generation of

reactive oxygen intermediates (ROIs) as a key event and results in the activation of NF- κ B (for review see 7, 8). The two major forms of I κ B proteins, termed I κ B- α and I κ B- β , can be inducibly phosphorylated and ubiquitinated (9-11). These posttranslational modifications tag the molecule for the subsequent proteolytical degradation by the ubiquitin-26S proteasome pathway (12-14). This induced degradation of I κ B proteins unmasks the nuclear localization sequences of the DNA-binding subunits of the NF- κ B dimer and allows NF- κ B to enter the nucleus, to bind to its DNA sequence and to induce transcription. The target genes whose transcription is mainly regulated by NF- κ B include many cytokines, cell adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), as well as acute-phase proteins and immunoreceptors (for review see 15).

Among its many different biological activities NF- κ B seems to play an important role in cell killing. NF- κ B has been shown recently to counteract the induction of apoptosis by the cytokine TNF- α , ionizing radiation and the cancer chemotherapeutic agent daunorubicine (for review see 16). However, there is also evidence for apoptosis-promoting properties of NF- κ B. Glutamate was found to induce NF- κ B in neuronal cells and acetylsalicylic acid (Aspirin[®]) protected these cells from NF- κ B-induced cell death (17, 18). Along this line, the overexpression of the cellular and viral anti-apoptotic proteins Bcl-2 and E1B 19 K both negatively interfered with the activation of NF- κ B under these conditions (19-21).

The role of NF- κ B in the immune response is also evident from gene disruption experiments. The targeted deletion of the p50, p65, RelB and c-Rel subunits resulted in an impaired immune response and/or in a reduced viability of the mice (22-26). These findings and the immunological relevance of most of the NF- κ B target genes make this transcription factor an interesting therapeutical target for the identification of inhibitors. One group of NF-

κ B inhibitors exerts its inhibitory effects by scavenging ROIs. These inhibitors include N-acetyl-L-cysteine (27, 28), pyrrolidine dithiocarbamate (29), acetylsalicylic acid (30, 31) or curcumin (32). All these compounds are structurally unrelated, but share the property of being anti-oxidative. Another group of inhibitors interferes with the induced degradation of I κ B-family members by affecting the functioning of the 26S proteasome (14, 33, 34). Further inhibitors of NF- κ B exert their effects only in the cell nucleus by impairing the transcriptional activity of NF- κ B already bound to DNA. Examples are inhibitors of the p38 MAP kinase (35, 36) and, at least in some cell types, glucocorticoids. Here, the activated glucocorticoid receptor directly binds to the NF- κ B dimer and thereby prevents transcriptional activation (37).

WO 96/25666 (PCT/US96/02122) describes a polypeptide I κ B- β which binds to and effects NF- κ B gene activation. Also disclosed is a nucleotide sequence encoding I κ B- β and methods of identifying compositions which affect I κ B- β /NF- κ B complexes. WO92/20795 (PCT/US9204073) describes the purification, cloning, expression and characterization of I κ B.

Summary of the invention

It has been found that SIs prevents the activation of NF- κ B by different stimuli; more precisely it has been found that treatment of cells with SIs prevented the induced degradation of I κ B- α and I κ B- β by these stimuli, suggesting that they interfere with a common step in the activation of factor NF- κ B.

We recently identified SLs isolated from extracts of Mexican indian medicinal plants as specific inhibitors of NF- κ B (1). SL-containing plant extracts are frequently used in the

traditional Mexican indian medicine for the treatment of infections of the skin and other organs (for review see 38). The SL parthenolide is also contained in drugs such as Feverfew® (*Tanacetum parthenium*) used against migraine, an illness that has been implicated with neurogenic inflammatory processes (39). The anti-inflammatory activity of the SL-containing plant extracts was confirmed in the hen egg tests where they showed a delay in cell culture experiments (40) and the onset of capillary reactions of the allantois membrane (1).

The present invention shows that SLs prevent a common step in NF- κ B activation. They did not interfere with the generation of oxygen radicals, but prevented the induced degradation of I κ B- α and I κ B- β as well as the induced expression of the NF- κ B target gene ICAM-1. Structural studies identified the exomethylene group in conjugation with the lactone group as the decisive structural feature for the inhibitory activity.

Numerous biological activities have been reported for SLs, including antimicrobial (48), antiviral (49) and antitumor activities (50). Furthermore SLs or SL-containing plant extracts were found to have anti-inflammatory properties (51, 52). Anti-phlogistic activities were also seen in hen eggs assays, in the reduced production of the inflammatory cytokine IL-6 and in cell culture experiments (1, 40). It was previously reported that the anti-inflammatory effects of SLs can be assigned -at least to a certain extent- to the inhibition of transcription factor NF- κ B, a central mediator of the immune response (1). The present invention shows that SLs do not interfere with the generation of ROIs following the stimulation of cells, but prevent the induced degradation of I κ B- α and I κ B- β . The SLs do not directly act on the DNA-binding subunits of NF- κ B. Also the I κ B subunits seems not to be a direct target for the SLs. The incubation of cell extracts from unstimulated cells with SLs did not change the

inducibility of NF- κ B by the dissociating agent desoxycholate (M.L.S., unpublished results). Furthermore the re-synthesis of the putative target protein(s) after more than 18 h makes the I κ B proteins an unlikely candidate for the SLs, since I κ B- α is completely resynthesized within one hour (53).

Various protein kinases have been implicated in the induced activation of NF- κ B, but the kinases participating in these signaling events remain poorly defined. The signal transducing events leading to NF- κ B include the small GTP-binding proteins Rac1 (54) and Cdc 42 (55) which then lead to the activation of mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK-1). The activation of MEKK-1, which has recently been shown to be necessary for the induction of NF- κ B (56), results in the induced activity of mitogen-activated protein kinase kinase (MKK4/SEK) and the c-Jun N-terminal kinase (JNK) (57). The kinase finally phosphorylating I κ B- α at serines 32 and 36 is called CHUK (58) or IKK (59). It is currently not clear which identified or so far unidentified member(s) of the signaling cascade is the target for SLs. Furthermore the number of molecules inhibited by SLs remains unknown.

The role of NF- κ B in the induction of cell death is still not yielding a homogenous picture. The inhibition of NF- κ B promotes TNF- α -mediated cell death in HeLa cells, macrophages, fibroblasts, fibrosarcoma cells and Jurkat cells (60-62), while it is ineffective in other cell lines such as human breast carcinoma cells (64). Part of the discrepancies may be explained by differences of the cell type studied and the nature of the apoptosis-inducing stimulus. This study shows that the incubation with parthenolide facilitates cell-killing by TNF- α in mouse L929 cells, although only additional experiments using further inhibitors of NF- κ B can really prove the protecting role of this transcription factor for L929 cells. The mechanism of this cell-protecting effect of NF- κ B is still not clear. It might rely on the induced expression

of anti-apoptotic genes such as the zinc finger protein A20 or manganese superoxide dismutase (for review see 16).

The SLs tested in this study display a high degree of specificity for their inhibitory activity, since they did not influence the activity of other transcription factors such as AP-1, RBP-J κ and Oct-1 (1; this study). Furthermore the SLs did not impair the activity of the T-cell kinases p59^{fyn} and p60^{src}. These results show that the SLs do not interfere in a non-specific manner with transcription factors or signaling molecules. A potential target-specificity of SLs may well be explained by considering the fact that the combination of the reactive Michael-acceptor system together with the oxygen-substituted isoprenoide rings forms a pattern of potential non-covalent binding sites (e.g. hydrogen bonds). These binding sites would allow the SLs to interact with complementary sites on the surface of the target molecule(s). The relative positions of SLs and other inhibitors of NF- κ B in the signaling cascade are schematically displayed in Figure 9. This allows the usage of this drug for the analysis and dissection of the diverse signal transduction pathways finally resulting in the activation of NF- κ B. Since the various classes of inhibitors interfere with NF- κ B activation at different levels in signal transduction it is tempting to speculate that a mixture of these different inhibitors might be highly effective in NF- κ B inhibition. Furthermore the individual doses required for an optimal blocking may presumably be reduced in such a blend, thereby reducing the respective side effects.

The inhibition of NF- κ B may be of therapeutic use for the treatment of chronic diseases such as rheumatoid arthritis or for the acute situations such as septic shock. There is emerging evidence for the role of NF- κ B during rheumatoid arthritis (for review see 2) and the importance of NF- κ B during Crohn's disease. Here, the local administration of antisense

phosphorothioate oligonucleotides to the p65 subunit of NF- κ B abrogated established experimental colitis in mice (65). Also other chronic inflammatory diseases such as Alzheimer's disease involve the activation of NF- κ B. The amyloid β peptide, which is a major component of the plaque, induces an increase in ROIs and activates the nuclear translocation of the p50 and p65 subunits of NF- κ B in the neurons directly surrounding the plaque (5). Moreover the septic shock syndrome is associated with a massive activation of NF- κ B. Septic shock occurs when microbial products such as LPS stimulate the expression of inflammatory cytokines. This massive production of cytokines leads to failure of circulation and general organ function. Therefore it is of therapeutical interest to develop drugs that are able to interfere with the activity of NF- κ B.

The complete inhibition of NF- κ B is lost only to 50 % after 18 h post treatment, suggesting an irreversible mechanism such as a covalent modification of proteins. Also other drugs such as Aspirin[®] and omeprazole (Antra[®]), lead to to an irreversible inactivation of their target molecules (for review see 66, 67). The covalent modification of a target protein by a drug reduces the required frequency of drug application.

The determination of the structural requirements of SLs for NF- κ B inhibition presented in this study provides the basis for a rational development of a new generation of anti-inflammatory substances interfering with NF- κ B.

This and other aspects of the present invention become more fully appreciated upon consideration of the invention described below.

Brief description of the drawings

Fig. 1. SLs inhibit NF- κ B activation by different stimuli.

Fig. 2. SLs do not interfere with DNA-binding of activated NF- κ B.

Fig. 3. Effects of SLs on DNA-binding of Oct-1 and the fragmentation of I κ B- α .

Fig. 4. SLs inhibit the degradation of I κ B- α and I κ B- β induced by various stimuli.

Fig. 5. Reversibility of SL treatment.

Fig. 6. Effect of parthenolide on the cell killing by TNF- α .

Fig. 8. SLs do not change the intracellular redox-state.

Fig. 9. Distinct positions of NF- κ B inhibitors in the activation cascade.

Detailed description of the preferred embodiments

Fig. 1 A, shows the inhibition of NF- κ B activated by H₂O₂. Jurkat JR cells were preincubated with the indicated amounts of parthenolide for one h and stimulated with various concentrations of H₂O₂ for 90 min. Subsequently total cell extracts were prepared and tested for DNA-binding of activated NF- κ B by EMSA. B shows the inhibition of NF- κ B activated by CD3/CD28 ligation. Jurkat J16 cells were preincubated with the indicated amounts of parthenolide for 1 h and activated by immobilized α -CD3 antibodies and crosslinked α -CD28 antibodies. Ten minutes after stimulation cells were harvested and assayed for NF- κ B activity by EMSA. The SLs were kept with the cells during both stimulations. The filled arrowhead indicates the location of the DNA-NF- κ B complex, the circle indicates the position of a constitutively DNA-binding protein and the open arrowhead points to the unbound oligonucleotide.

Fig. 2. HeLa cells were stimulated for 20 min with PMA and total cell extracts of the stimulated cells were pooled. These extracts were incubated for 1 h with various concentrations of parthenolide as indicated. Subsequently these extracts were tested together with a protein extract

from unstimulated HeLa cells as a control for DNA-binding activity of NF- κ B by EMSA. Bound and free oligonucleotides were separated by electrophoresis on a native gel, dried and exposed. The symbols used are as explained in Fig. 1.

Fig. 3. Mouse L929 fibroblasts were preincubated with parthenolide for 1 h prior to stimulation with murine TNF- α . Subsequently cell extracts from these cells were prepared, together with extracts from unstimulated and TNF- α -stimulated cells as indicated. Equal amounts of the protein extracts were simultaneously tested for DNA-binding activity of NF- κ B (A), Oct-1 (C) and for the presence of I κ B- α in Western blots (B). A, DNA-binding activity of NF- κ B. Cells were treated as indicated and total cell extracts were tested for NF- κ B binding by EMSA. The symbols used are as described for Fig. 1. B, I κ B- α Western blot. Total cell extracts were separated by SDS-PAGE and transferred to a PVDF membrane. The arrow points to the I κ B- α protein which was detected with an α -I κ B- α antibody. The positions of prestained protein markers are indicated. C, DNA-binding activity of Oct-1. The indicated cell extracts were tested for the activity of the constitutively DNA-binding protein Oct-1 by EMSA. The open arrowhead points to the position of the unbound oligonucleotide, the filled arrowhead indicates the position of the DNA-protein complex.

Fig. 4. Cells were preincubated with 10 μ M of parthenolide 1 h prior to stimulation with 2000 U TNF- α (A), 50 ng/ml PMA (B), ligation of the CD3/CD28 receptors (C) and 100 μ M of H₂O₂ (D). Cells were harvested at the indicated time points and total cell extracts were prepared. Equal amounts of protein were tested by EMSAs for DNA-binding of NF- κ B and by Western blot experiments for the occurrence of I κ B proteins. The shifted DNA-protein complexes are shown in

the upper parts of the respective panels with the filled arrowhead pointing to the position of the NF- κ B-DNA complex. The same extracts were simultaneously tested for the occurrence of I κ B- α and I κ B- β in Western blots with the arrows pointing to the respective I κ B proteins. Representative experiments are shown, for further details see text.

Fig. 5. HeLa cells were preincubated with 5 μ M of parthenolide for 1 h. Subsequently the medium was replaced by parthenolide-free medium and the cells were grown for the indicated time periods prior to stimulation with PMA for 15 min. NF- κ B activity was then assessed by EMSA and the amount of DNA-bound NF- κ B was quantitated in a PhosphorImager (Molecular Dynamics). A, schematic outline of the experimental strategy. The incubation time with parthenolide is symbolized by the black columns, the stimulation period is highlighted by hatching. B, time-dependence of NF- κ B inhibition. The diagram shows the percental inhibition of NF- κ B activity in dependence of the time period between parthenolide preincubation and PMA-stimulation. The complete inhibition of NF- κ B in the experiment without incubation in parthenolide-free medium was set as 100%. A typical experiment is shown.

Fig. 6. L929 cells were incubated with either 5 μ M parthenolide (\blacklozenge), 2000 U/ml TNF- α (σ), or a combination of both (\vee). At the indicated time points cell viability was determined using the MTT assay. Viable cells remaining after treatment with mTNF- α are shown as a percentage of viable untreated cells. A representative experiment is shown.

Fig. 7. A, effect of TNF- α on the expression of ICAM-1. Jurkat JR cells were stimulated for 10 h with 2000 U/ml of TNF- α , stained with an α -ICAM-1 antibody coupled to phycoerythrine and

analyzed by flow cytometry. The profiles of untreated (white) and TNF- α -stimulated (black) cells are shown. B, effect of parthenolide on ICAM-1 expression. Jurkat cells were treated as in (A) in the presence of 10 μ M parthenolide. Profiles of unstimulated (white) and stimulated (black) cells are displayed.

Fig. 8. A, effects of TNF- α on the concentration of intracellular ROIs. Jurkat JR cells were either left untreated or stimulated for 15 min with human TNF- α (2000 U/ml). Subsequently ROIs were measured by FACS analysis of DFCH-stained cells. FACS profiles of untreated (white) and TNF- α -stimulated cells (dark) are shown. B, effect of parthenolide on the cellular redox-state. Jurkat T-cells were incubated with 5 μ M parthenolide and further treated and analyzed as described in part (A) of this figure. FACS profiles of untreated (white) and TNF- α -stimulated cells (dark) are shown. C, comparison of ROI amounts. The mean fluorescence of unstimulated cells was set as 1 and directly compared to the fluorescence of the TNF-stimulated cells in the presence or absence of parthenolide as indicated.

Fig. 9. Diverse stimuli of NF- κ B are given on the top. The various classes of NF- κ B inhibitors are shown in boxes. Abbreviations not used in the text are: B7-1: B-cell activation antigen, PKC: protein kinase C. TCR: T-cell receptor. Further details are given in the text.

Experimental Procedures

Cell culture

Jurkat T leukemia cells (subclone JR „Würzburg“) were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS and 1% (w/v) penicillin/streptomycin (all purchased from GIBCO Laboratories, Gran Island, NY). HeLa cells and L929 fibroblasts were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% FCS and 1% (w/v) penicillin/streptomycin. All cells were grown in an incubator at 37°C and 5% CO₂. TNF- α and poly (dI-dC) were obtained from Boehringer Mannheim (Mannheim, Germany). Parthenolide, isohelenin, isophoronoxide, limonenoxide, caryophyllenoxide, sclareolide and santonin were from Sigma Inc. (St. Louis, MO). Antibodies directed against I κ B- α and NF- κ B were from Santa Cruz Inc. (Santa Cruz, CA), α -CD28 antibodies were obtained from Pharmingen Inc. (San Diego, CA) and α -CD3 antibodies were isolated from a hybridoma cell line. The phycoerythrine-conjugated antibody against ICAM-1 was obtained from Dianova (Hamburg, Germany). All other chemicals were either from Sigma, Aldrich (Steinheim, Germany) or Roth (Karlsruhe, Germany).

Electrophoretic mobility shift assay (EMSA)

HeLa or L929 cells (5×10^5) were grown overnight on 10 cm dishes, Jurkat cells (approximately 1×10^6 /ml) in cell culture flasks. One hour prior to stimulation by either TNF- α , phorbol-12-myristate 13-acetate (PMA) or hydrogen peroxide, cells were preincubated with the indicated amounts of the tested substances for 60 minutes at 37°C. The tested substances were dissolved in dimethyl sulfoxide (DMSO) as a solvent. In the following cells were stimulated for 20 min with PMA at a final concentration of 50 ng/ml, TNF- α (2000 U/ml), α -CD3/ α -CD28 (1 μ g/ml) antibodies or for the indicated periods with the specified amounts of hydrogen peroxide. Cells were harvested by centrifugation and washed twice with cold TBS buffer (25 mM Tris/HCl pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM CaCl₂, 0.1 mM MgCl₂). The pellet was resuspended in TOTEX buffer (20 mM Hepes/KOH pH 7.9, 0.35 M NaCl, 20% (v/v) glycerol, 1% (v/v) NP-40,

1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF)) and incubated on ice for 30 min. The samples were carefully vortexed every 10 min. The cell debris was pelleted upon centrifugation with 14000 rpm at 4°C for 10 min. Equal amounts of supernatant were tested for DNA binding activity as described in detail elsewhere (1). Briefly, the extracts were incubated with 2 µg poly (dI-dC), 2 µg BSA and 10000 cpm of a ³²P labeled oligonucleotide on ice in 5 x binding buffer (20% (w/v) Ficoll 400, 100 mM Hepes/KOH pH 7.9, 1 mM DTT and 300 mM KCl) in a final volume of 20 µl. Subsequently the free and the oligonucleotide-bound proteins were separated by electrophoresis on a native 4% polyacrylamide gel. The gel was dried after electrophoresis and exposed to an X-ray film (Amersham Hyperfilm). The following oligonucleotides (binding site underlined) were used:

NF-κB: 5'-AGTTGAGGGGACTTTCCCAGGC-3'

3'-TCAACTCCCCTGAAAGGGTCCG-5'

Oct-1: 5'-TGTCGAATGCAAATCACTAGAA-3'

3'-ACAGCTTACGTTTAGTGATCTT 5'

Western Blotting

Cell extracts were separated on a 12 % reducing SDS polyacrylamide gel. Subsequently the proteins were transferred from the SDS gel onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) using a semi-dry blotting apparatus (Bio-Rad laboratories, Munich, Germany). Transfer efficiency was monitored by Ponceau S staining of membranes. Prior to the incubation with the α-IκB-α and IκB-β antibodies (Santa Cruz Inc. Santa Cruz, CA), the membrane was blocked with 5% non-fat dry milk powder in TBST buffer (25 mM Tris/HCl pH

7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM CaCl₂, 0.1 mM MgCl₂, 0.05 % (v/v) Tween-20). The membrane was then incubated in a small volume of TBST containing an 1:1000 dilution of the α -I κ B antibodies. After an overnight-incubation the membrane was extensively washed in TBST buffer and incubated for another hour in TBST containing a 1:3000 dilution of α -rabbit antibody coupled to horseradish peroxidase. After extensively washing the membrane the immunoreactive bands were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Lifescience, Braunschweig, Germany).

In vitro kinase assays

The Src family protein tyrosine kinases p60^{src} and p59^{lyn} were expressed in baculovirus-infected Sf9 cells and purified by affinity chromatography as described (41). The effect of SLs on protein kinase activity was determined using enolase as a substrate. Briefly, serial concentrations of parthenolide and isohelenin were preincubated at 30°C with p60^{src} or p59^{lyn} protein in 50 μ l kinase buffer (30 mM Hepes pH 7.2, 5 mM MgCl₂, 5 mM MnCl₂, 1 μ M ATP, 10 μ Ci ³²P- γ -ATP) containing 5 μ g of acid-treated rabbit muscle enolase (Sigma Inc., St. Louis, MO). After 15 min, the reaction was ended by the addition of Laemmli buffer. The samples were analyzed by SDS-PAGE and subjected to autoradiography.

Cell killing assays

The cytotoxic activity of TNF was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT assay) essentially as described by Mosmann (42). L929 cells were seeded at a density of 1×10^4 cells per well in 96-well microtiter plates (flat bottomed) and incubated for 16 h in 0.2 ml culture medium. The supernatant was then removed and replaced by fresh medium containing TNF (2000 U/ml) or/and parthenolide (5 μ M). At the

indicated times 20 μ l of a MTT solution (5 mg/ml PBS) was added to all wells. After another 3 h incubation supernatants were removed followed by addition of 100 μ l of a 24:1 (v/v) isopropanol/HCl solution. After 15 min at room temperature the absorbance of each well was determined with an automated plate reader (Digiscan, Asys Hitech, Austria) at 550 nm.

FACS analysis

The intracellular formation of reactive oxygen intermediates was measured using dichlorofluorescein-diacetate (DFCH) according to Royall and Ischiropoulos (43). Briefly, a 10 mM stock solution of DFCH (Molecular Probes, Eugene, OR) was prepared in DMSO under nitrogen and stored at -20°C. Cells were incubated with 5 μ M DFCH in phosphate-buffered saline (PBS) for 0.5 h at 37°C. ICAM-1 expression on Jurkat cells was determined using a FITC-conjugated anti-CD 54 (ICAM-1)-antibody (Dianova, Hamburg, Germany). In both cases measurements were performed in duplicates using a FACScan (Becton Dickinson, Heidelberg, Germany) flow cytometer. Dead cells were excluded by forward/side scatter gating and staining with propidium iodide.

Example 1

SLs inhibit a common step in NF- κ B activation

From a previous study it was evident that SLs prevent the PMA-induced activation of NF- κ B

(1). Therefore we studied whether SLs can inhibit NF- κ B activation in response to stimuli different from PMA. As an example for a receptor-mediated pathway mouse L929 cells were stimulated with TNF- α with or without preincubation with various amounts of isohelenin or

parthenolide. After 1 h of preincubation cells were stimulated for 20 min with murine TNF- α and total extracts were tested for DNA-binding activity by EMSA. The TNF-induced binding of NF- κ B was completely prevented by preincubation of cells with 5 μ M of parthenolide and 20 μ M of isohelenin, respectively (data not shown). Since it is known that most inducers of NF- κ B lead to the formation of ROIs, we tested whether SLs would interfere with H₂O₂-induced NF- κ B binding activity. Therefore Jurkat JR cells were preincubated for 1 h with various amounts of parthenolide and isohelenin and treated with various concentrations of H₂O₂ for 90 min. The extracts prepared from these stimulated cells were subsequently analyzed by EMSA (Fig. 1 A). Only H₂O₂ concentrations between 100 and 250 μ M activated NF- κ B, whereas the addition of 1 mM H₂O₂ failed to efficiently induce NF- κ B probably due to an oxidative destruction of the protein. The H₂O₂-induced DNA-binding activity of NF- κ B was completely prevented by preincubation with the two SLs in low micromolar concentrations. Another stimulus of NF- κ B with special relevance in T-cells is the activation of the CD3/CD 28 pathway, which also leads to an increased concentration of ROIs (44). T-cell specific activation of NF- κ B in Jurkat T-cells by crosslinking the CD3- and CD28-receptors was prevented by preincubation with low micromolar concentrations of the two SLs (Fig. 1 B). All these experiments indicate that SLs interfere with one or more common steps during NF- κ B activation in different cell types rather than with one single event specific for an individual stimulus. They are therefore likely to display their inhibitory properties after the point of integration of the different signals.

Example 2

SLs do not affect activity of Src family protein tyrosine kinases

Tyrosine kinases of the Src family have been implicated in NF- κ B activation in response to various stimuli including UV radiation, T-cell receptor ligation and stimulation with prooxidants. We therefore investigated the effect of parthenolide and isohelenin on kinase activity of recombinant p60^{src} and p59^{lyn}. After pretreatment with various concentrations of SLs, kinase activities were determined by incubation of the protein kinases with the substrate rabbit muscle enolase and ³²P- γ -ATP. Tyrosine-phosphorylated proteins were then separated by SDS-PAGE and visualized by autoradiography. Neither increasing amounts up to 20 μ M of isohelenin nor of parthenolide did affect the ability of p59^{lyn} to phosphorylate itself or the substrate enolase. Also the recombinant p60^{src} kinase was completely unchanged in its phosphorylating activity in the presence of both SLs (data not shown). These results suggest that SLs do not prevent NF- κ B activation by inhibiting Src tyrosine kinases. Furthermore these data indicate that SLs do not unspecifically alter the activity of cytoplasmatic enzymes by reacting with the sulfhydryl group of cysteine residues, because both Src family tyrosine kinases possess exposed and redox-reactive cysteines which were shown to be important for their activity (45).

Example 3

SLs do not prevent DNA-binding of NF- κ B

In order to investigate the mechanism of action of SLs we first tested the potential effects of the SLs parthenolide and isohelenin on the DNA-binding activity of NF- κ B in band-shift experiments. Therefore HeLa cells were stimulated with PMA and cell extracts were prepared 20 min after stimulation. These extracts, which contain the activated nuclear form of NF- κ B, were pooled and incubated either with various concentrations of the two SLs or with the

solvent DMSO as a control. The preincubated extracts were analyzed for DNA-binding activity of NF- κ B in an EMSA. Increasing concentrations of parthenolide completely abrogating NF- κ B activation in the intact cell (see Fig. 1) did not influence DNA-binding of activated NF- κ B in vitro (Fig. 2). The same amounts of isohelenin also failed to reduce the DNA-binding activity of NF- κ B (data not shown). This experiment excludes that SLs directly inhibit the DNA-binding activity of activated NF- κ B, e.g. by modifying reactive amino acids such as cysteine residues in the DNA-binding or dimerisation domains. The induced DNA-binding complex was confirmed to be a p50/p65 NF- κ B dimer by competition assays with unlabeled oligonucleotides and antibody reactivity (data not shown).

Example 4

SLs prevent the induced degradation of I κ B- α and I κ B- β

The part of the NF- κ B activation cascade that is influenced by SLs was further analyzed by following the fate of I κ B proteins in Western blots. Mouse L929 cells were preincubated for 1 h with 5 μ M of either isohelenin or parthenolide. Subsequently murine TNF- α was added without changing the medium and kept for 20 min on the cells. Total cell extracts were tested for the presence of I κ B- α in Western blots and simultaneously for DNA-binding of NF- κ B and Oct-1 by EMSAs (Fig. 3). The TNF- α -induced DNA-binding of activated NF- κ B in the EMSA experiment coincided with the degradation of I κ B- α as detected by Western blotting (Fig. 3 A,B). Preincubation of cells with 5 μ M parthenolide completely prevented the induction of the DNA-binding form of NF- κ B and protected I κ B- α from proteolysis by the 26S-proteasome. Identical results were obtained for HeLa cells preincubated with 5 μ M

isohelenin (data not shown). In a control experiment the DNA-binding activity of the Oct-1 protein remained unchanged (Fig. 3 C).

In a more detailed analysis we tested the behaviour of I κ B- α and I κ B- β proteins in the presence or absence of SLs with different NF- κ B-inducing conditions at various time points (Fig. 4 A-D). The rapid TNF- α -induced DNA-binding of NF- κ B in L929 cells remained unchanged up to 120 min (Fig. 4 A). Preincubation with parthenolide completely prevented the induced degradation of I κ B- α and I κ B- β as monitored by Western blotting. Sixty minutes after the addition of TNF- α only the I κ B- α protein was resynthesized. The PMA-induced DNA-binding of NF- κ B in HeLa cells took place with a significantly slower kinetic when compared to TNF- α . DNA-binding was complete thirty minutes after stimulation with PMA and resulted in a full degradation of I κ B- α , which was not apparent in the presence of parthenolide (Fig. 4 B). The I κ B- β protein was not inducibly degraded by PMA, suggesting the importance of alternative PMA-signaling pathways for I κ B- β . The effects of CD3/CD28 receptor ligation on NF- κ B and I κ B proteins were monitored in peripheral blood lymphocytes. Various time points after receptor ligation total cell extracts were prepared and tested for DNA-binding activity and I κ B degradation. The DNA-binding of NF- κ B was significantly enhanced already 5 min after receptor triggering. The observed constitutive DNA-binding activity is most likely due to the constitutively active form of NF- κ B from mature B-cells, which are contained in the peripheral blood lymphocytes. The stimulation of NF- κ B by CD3/CD28 ligation is reflected by a degradation of the I κ B- α and I κ B- β proteins, which again can be prevented by preincubation with parthenolide (Fig. 4 C). The activation of NF- κ B by 100 μ M of H₂O₂ in Jurkat JR cells appeared only after an incubation period of 90 minutes. As already seen for the other tested stimuli, the (in the case of H₂O₂ incomplete) degradation of

I κ B- α and I κ B- β proteins was abrogated by preincubation with parthenolide (Fig. 4 D). Identical results were obtained for all the stimuli described here with the SL isohelenin (data not shown). These experiments indicate that SLs prevent the induced degradation of I κ B- α and I κ B- β by diverse stimuli and therefore interfere with a common step in the signaling cascade leading to the activation of NF- κ B.

Example 5

SLs irreversibly inhibit NF- κ B activation

We next tested whether SLs act as competitive or irreversible inhibitors. HeLa cells were incubated with 5 μ M of parthenolide for 1 h. Subsequently the cells were washed with medium void of SLs and further grown for various periods as schematically displayed in Figure 5 A. After stimulation with PMA for 20 min cells were lysed and the extracts were assayed for NF- κ B activity by EMSA. The amount of DNA-bound NF- κ B dimers was quantitated with a phosphoimager and the results are displayed graphically (Fig. 5 B). The total inhibition of NF- κ B binding activity seen after immediate stimulation of cells following the preincubation with parthenolide was set as 100 %. The inhibition was still almost complete after 2 h of incubation and decreased to approximately 50 % after 18 h of incubation in parthenolide-free medium (Fig. 5 B). This kinetic behaviour suggests that the SLs act by covalently and thus irreversibly modifying their target molecule(s), presumably by their reactive Michael system in the lactone ring. The partial restauration of inducible NF- κ B

activation, which is occurring 18 h after SL-treatment, is most probably due to the re-synthesis of the inactivated protein(s).

Example 6

SLs promote killing of mouse L929 cells by TNF- α

The role of NF- κ B during the TNF- α -induced cell death is still not clear and seems to depend on the tested cell line (for review see 16). We therefore wanted to address the question whether cell death of L929 cells in response to TNF- α is influenced by parthenolide. Mouse L929 cells were incubated either with 5 μ M parthenolide or 2000 U/ml TNF- α alone or by a combination of both. After various incubation times the cell viability was measured (Fig. 6). Treatment with parthenolide alone did not influence the cell viability and TNF- α -induced cell death was enhanced in the presence of parthenolide. This experiment shows that the NF- κ B inhibitor parthenolide is also enhancing the TNF- α -induced cell killing of mouse L929 cells.

Example 7

SLs prevent the induced expression of the NF- κ B target gene ICAM-1

The inducible transcription of the ICAM-1 gene in response to TNF- α , IL-1 β and PMA is controlled by a NF- κ B binding site in its promoter (for review see 47). This feature makes ICAM-1 surface expression a good read-out to test the effect of SLs on the expression of endogenous NF- κ B target genes. The induction of ICAM-1 gene expression and the appearance of the protein on the cell surface was analyzed by FACS. Treatment of Jurkat T-cells with TNF- α resulted in a strong induction of ICAM-1 expression, as displayed in Figure

7 A. Preincubation with either 5 μ M parthenolide almost completely prevented the induction of ICAM-1 synthesis (Fig. 7 B). In a control experiment parthenolide or isohelenin did not influence the amount of the T-cell receptor CD3 protein on the surface of Jurkat cells in the presence or absence of TNF- α (data not shown). These results indicate that the induced transcription of NF- κ B target genes is specifically inhibited by SLs.

Example 7

Structural determinants for the inhibitory activity of SLs

We next investigated the structural features of the SLs which confer inhibitory activity on NF- κ B activation pathways. Two structural hallmarks of SLs are an isoprenoide ring system and a lactone ring. In many cases this lactone ring contains a conjugated exomethylene group. Both groups together form a reactive Michael system which is a target for nucleophilic substrates, e.g. for cysteine residues in proteins. Various isoprenoide substances lacking either the lactone ring or the exomethylene group were tested for their effects on NF- κ B activation. The structure of these tested compounds is given in Table 1. None of these substances showed cytotoxic effects on HeLa cells at concentrations of 5 and 10 μ M after 10 h of incubation time. One hour after preincubation with 5 and 10 μ M of the respective drugs HeLa cells were stimulated for 20 min with PMA. Subsequently total extracts of these cells were tested by EMSA on NF- κ B activation. All tested isoprenoides that lacked either the lactone or the exomethylene group in the α position to the lactone function displayed no inhibitory effect on the pathway leading to the activation of NF- κ B (see Table 1). Another interesting structural element especially of parthenolide is its epoxide ring which is also a likely site for the addition of nucleophilic reagents. To investigate the importance of this epoxide ring we also tested

substances with this structural feature, even in combination with exomethylene groups to provide a bi-reactive substrate for (the) target molecule(s). Again none of the tested substances displayed inhibitory properties on NF- κ B as assessed by EMSA (Table 1). This failure of inhibition occurred irrespective of a synthetic or natural origin of the tested substances.

Example 8

SLs inhibit NF- κ B activation without having anti-oxidative properties

The chemical structure of the SLs suggests that they do not have anti-oxidative properties such as many other inhibitors of NF- κ B. In order to exclude that SLs display any direct or indirect anti-oxidative effects we measured the potential influence of parthenolide preincubation on the TNF- α induced change of the intracellular redox state in Jurkat T-cells. Quantitation of the intracellular levels of ROIs by FACS analysis with the dye DFCH demonstrated that preincubation of Jurkat T-cells for 1 h with 5 μ M of parthenolide did not reduce the amount of intracellular ROIs that were generated by TNF- α -stimulation (Fig. 8 A,B). This observation is confirmed by a direct comparison of ROI-concentrations under various conditions (Figure 8 C).

Example 9

Crystallography of the NF- κ B/ I κ B- α /I κ B- β /parthenolide complex

Co-Crystallization of the NF- κ B/I κ B- α or NF- κ B/I κ B- β complex in the presence of parthenolide revealed the structure of the parthenolide binding site on I κ B- α and I κ B- β . The

structural data as resulting from mass spectroscopic studies (results are not shown) are relevant for rational drug design.

Example 10

***In vivo* effect of sesquiterpene lactones**

Animal experiments have been designed to investigate the *in vivo* effect of sesquiterpene lactones, namely parthenolide and isohelenin, based on the above described mechanism.

Abbreviations

CHUK, conserved helix-loop-helix ubiquitous kinase; DFCH, dichlorofluorescein-diacetate; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assay; ICAM-1, intercellular adhesion molecule-1; IKK, I κ B kinase; JNK, c-Jun N-terminal kinase; MEKK-1, mitogen-activated protein kinase/ERK kinase kinase-1; MKK4, mitogen-activated protein kinase kinase 4; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; PMA, phorbol-12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; ROIs, reactive oxygen intermediates; SLs, sesquiterpene lactones; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule-1.

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Claims

1. Use of sesquiterpene lactones by binding $\text{I}\kappa\text{B-}\alpha$ and $\text{I}\kappa\text{B-}\beta$ thereby inhibiting the activation of $\text{NF-}\kappa\text{B}$.
2. Use of sesquiterpene lactones for drug design.
3. Use of sesquiterpene lactones as drugs.
4. Characterization of the the structure of the sesquiterpene lactone binding sites by co-crystallization of $\text{NF-}\kappa\text{B/I}\kappa\text{B-}\alpha$ or $\text{NF-}\kappa\text{B/I}\kappa\text{B-}\beta$ complexes with sesquiterpene lactones.
5. Characterization of the *in vivo* effect by sesquiterpene lactones using animal models.

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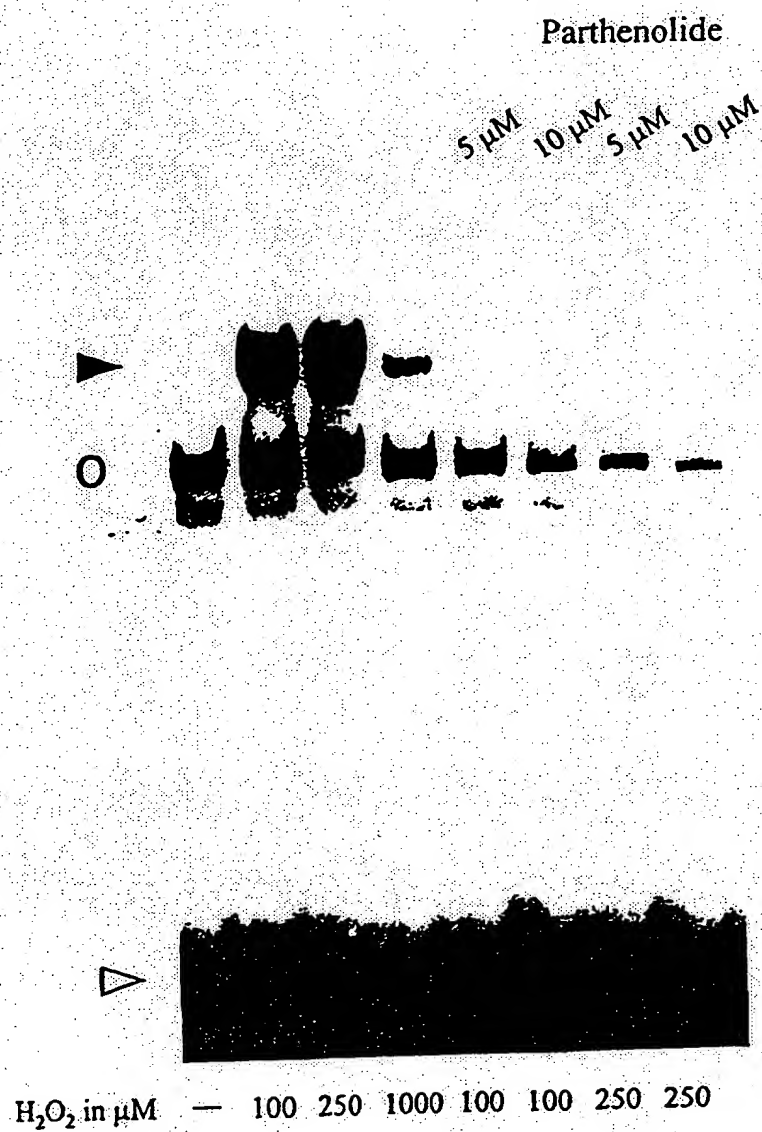


Fig. 1A

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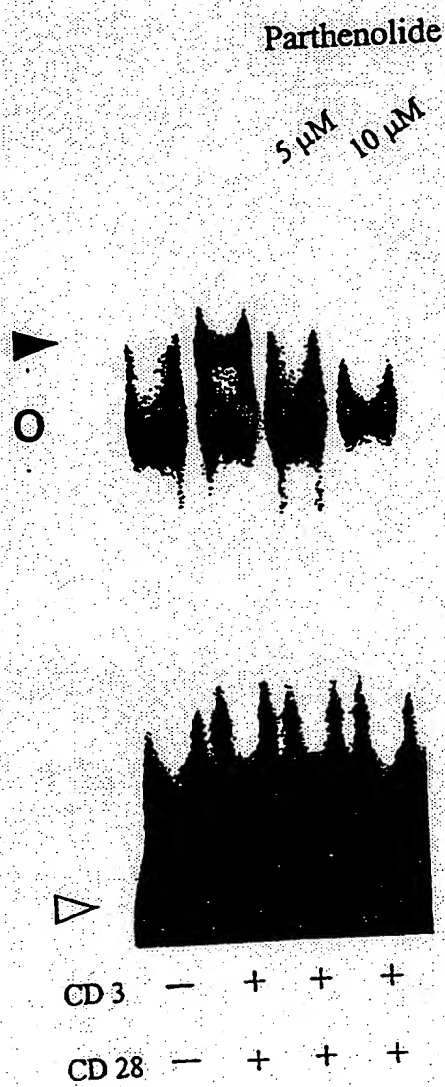


Fig. 1B

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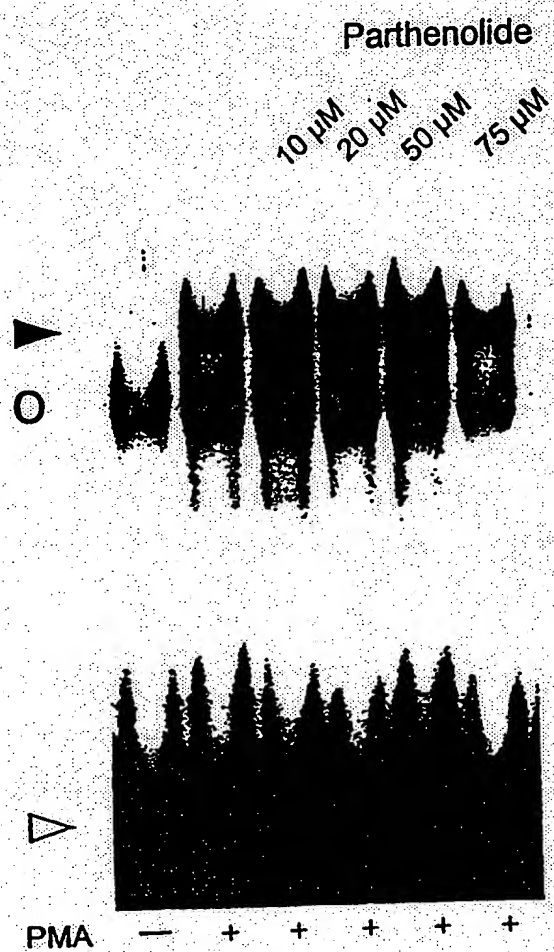


Fig. 2

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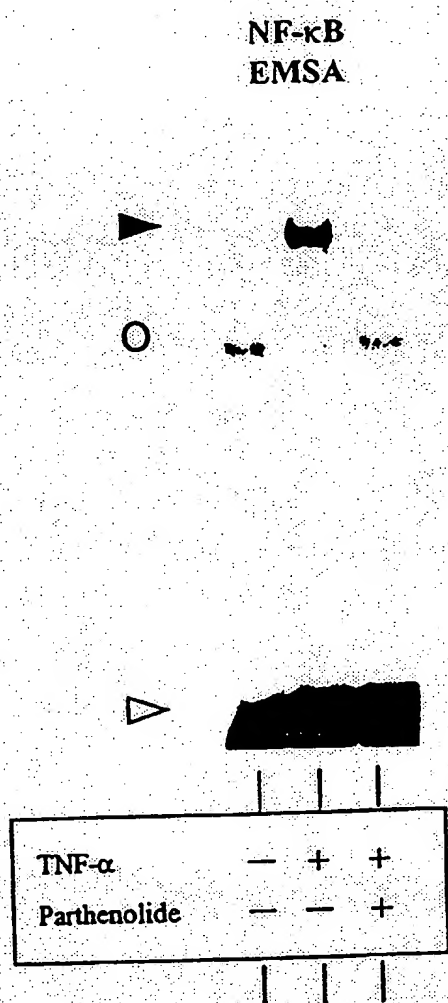


Fig. 3A

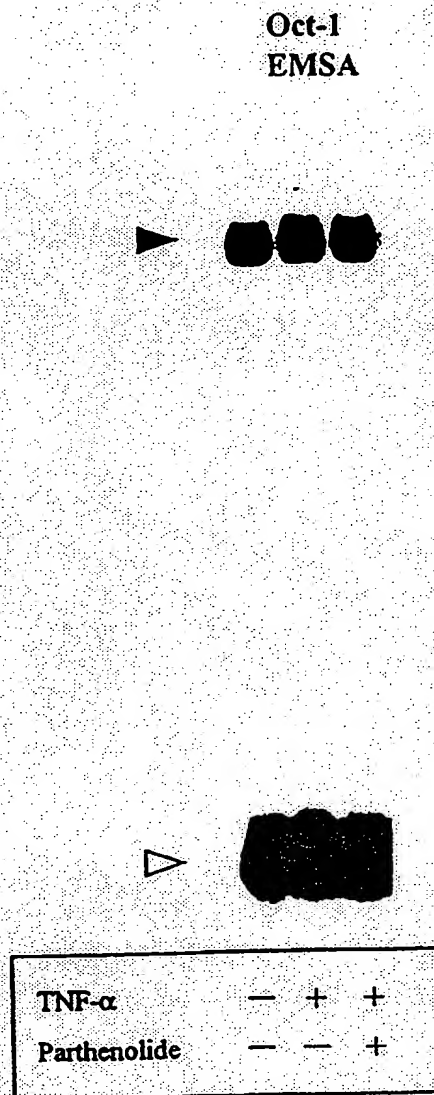


Fig. 3C



Fig. 3B

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TNF- α (min)	0	5	5	30	30	60	60	120	120
Parthenolide	-	-	+	-	+	-	+	-	+

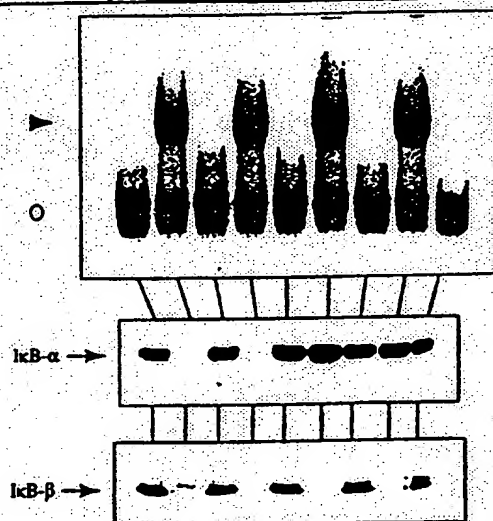


Fig. 4A

PMA (min)	0	5	5	30	30	60	60	120	120
Parthenolide	-	-	+	-	+	-	+	-	+

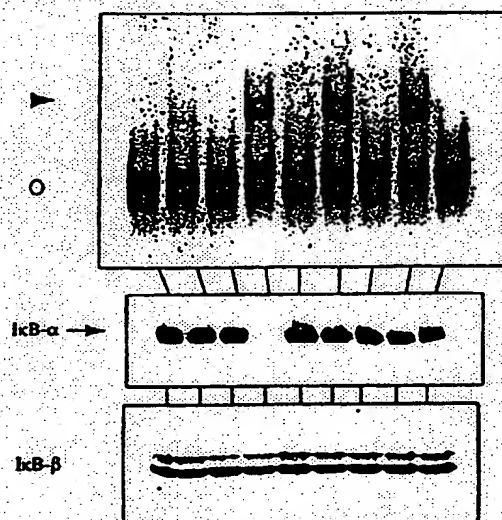


Fig. 4B

CD3/CD28 (min)	0	5	5	30	30	60	60	120	120
Parthenolide	-	-	+	-	+	-	+	-	+

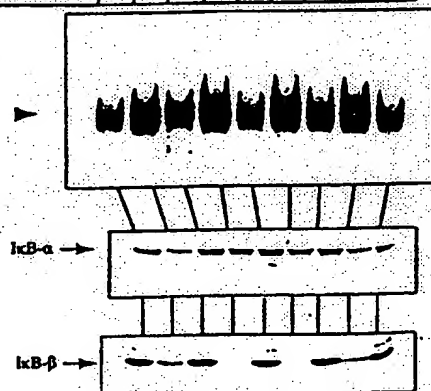


Fig. 4C

H ₂ O ₂ (min)	0	50	50	90	90
Parthenolide	-	-	+	-	+

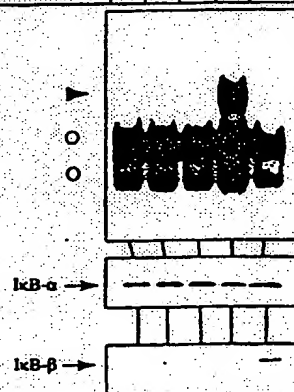


Fig. 4D

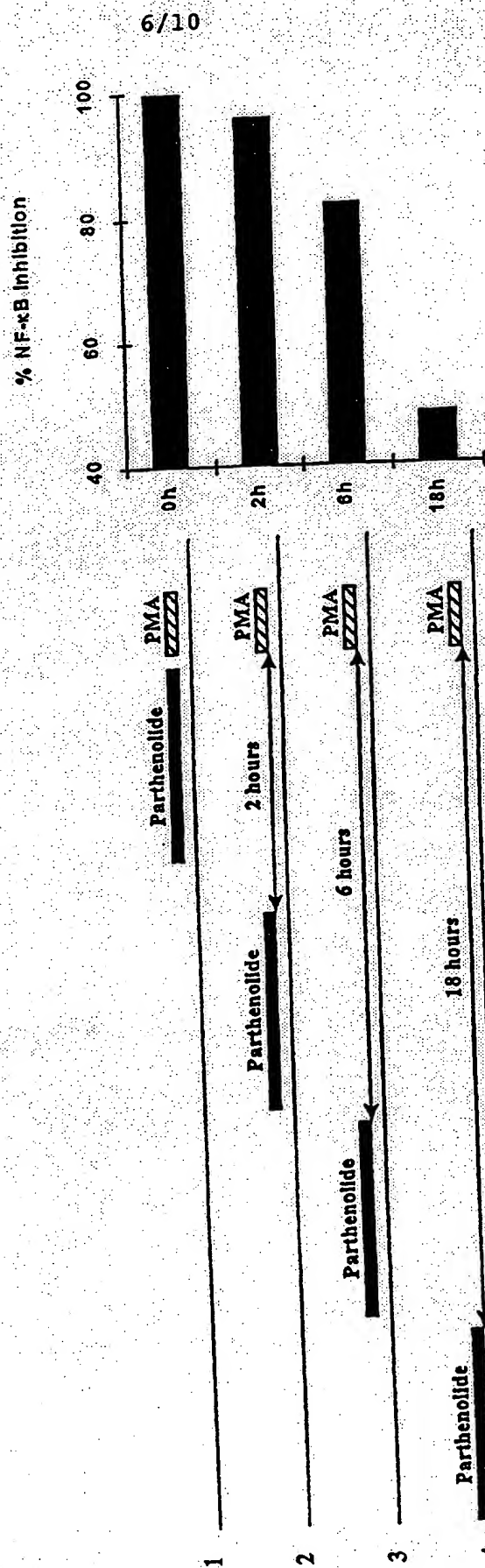


Fig. 5B

Fig. 5

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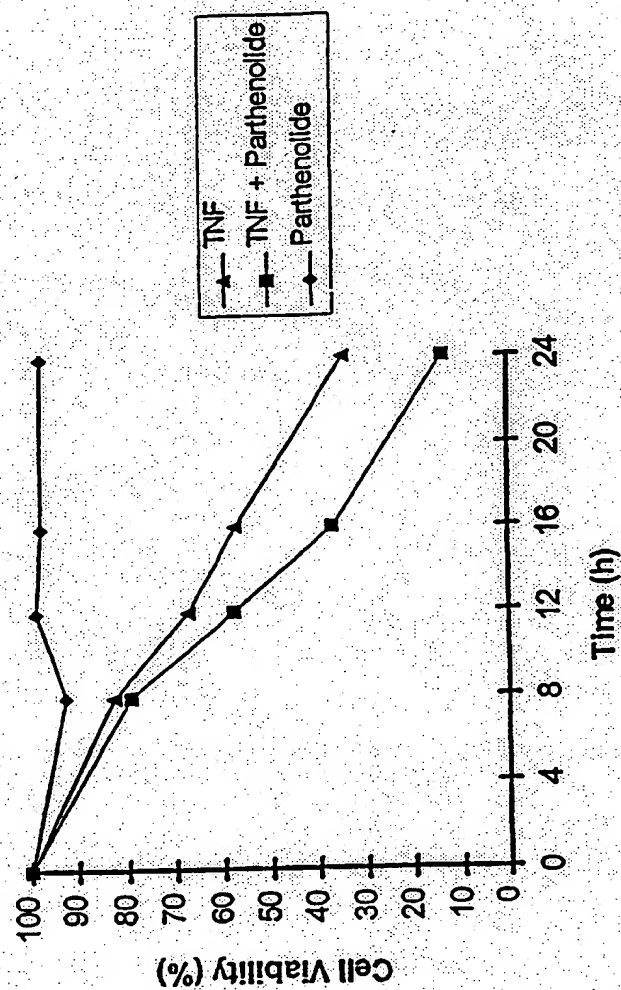


Fig. 6

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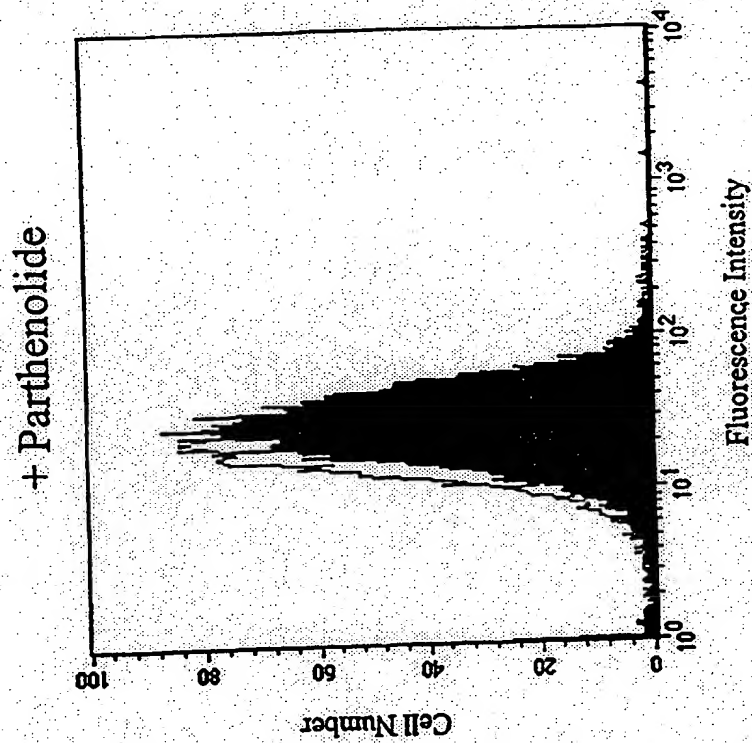


Fig. 7B

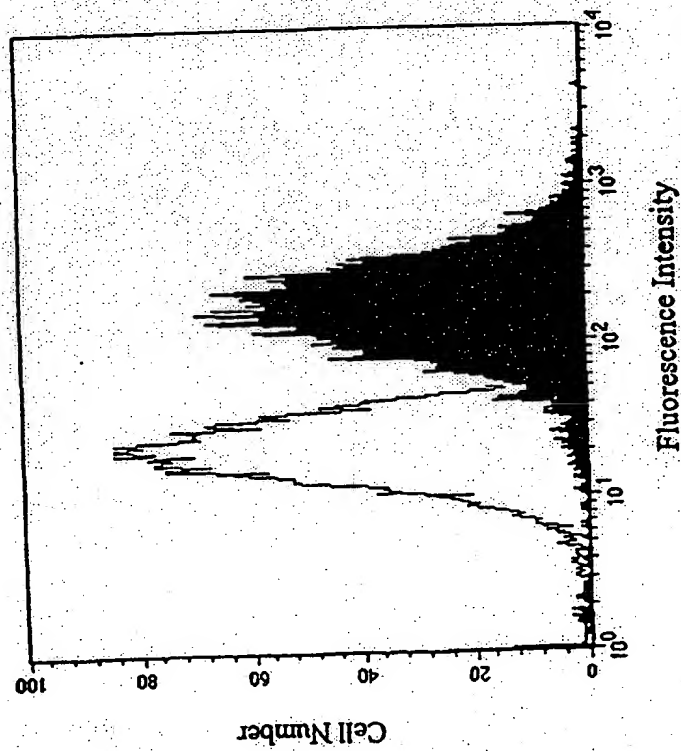


Fig. 7A

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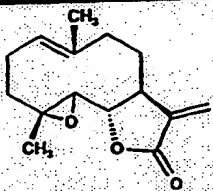
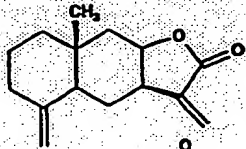
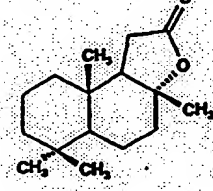
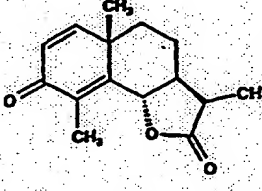
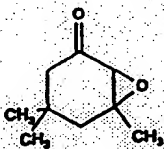
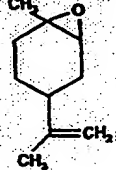
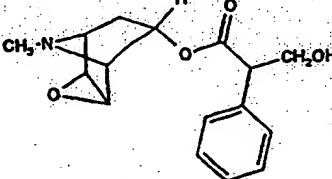
		NF- κ B Inhibition	
		after PMA stimulation	after TNF- α stimulation
Parthenolide		> 5 μ M	> 10 μ M
Isohelenin		> 10 μ M	> 20 μ M
Sclareolide		none up to 40 μ M	none up to 40 μ M
Santonin		none up to 100 μ M	none up to 100 μ M
Isophoronoxide		none up to 40 μ M	none up to 100 μ M
Limonenoxide		none up to 40 μ M	none up to 40 μ M
Scopolamine		none up to 40 μ M	none up to 40 μ M

Table 1. List of different substances tested for their inhibitory effect on the activation of NF- κ B after PMA or TNF- α stimulation.

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+ Parthenolide

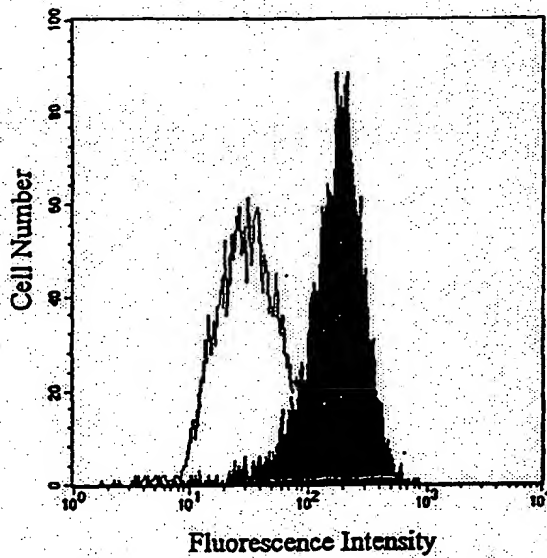


Fig. 9A

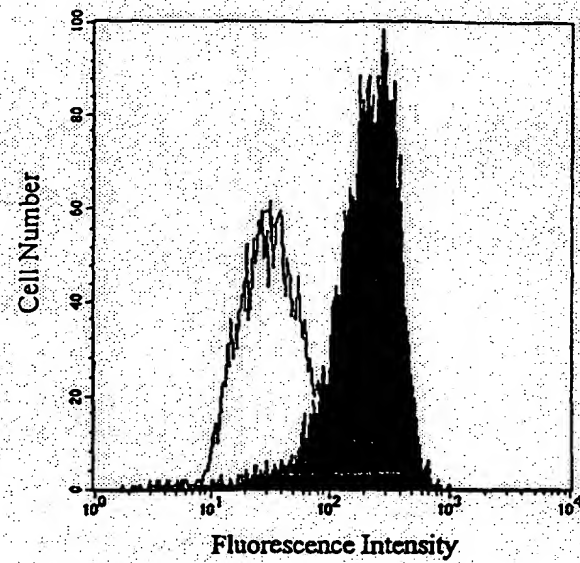


Fig. 9B

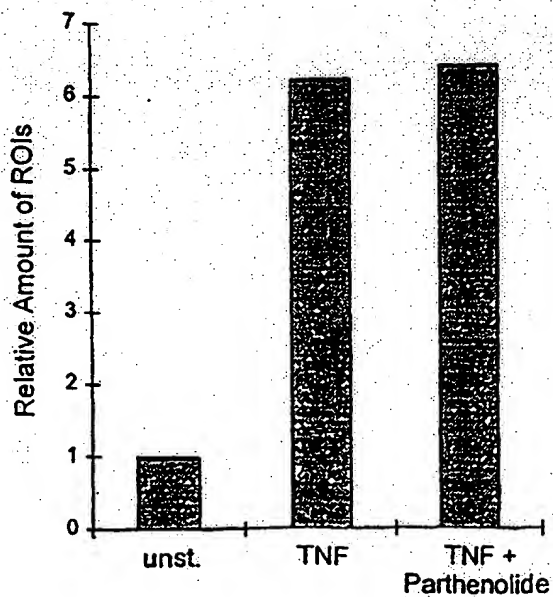


Fig. 9C

INTERNATIONAL SEARCH REPORT

In tional Application No
PCT/IB 98/02108

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/365 A61K31/78

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	P M BORK ET AL: "Sesquiterpene lactone containing Mexican Indian medicinal plants and pure sesquiterpene lactones as potent inhibitors of transcription factor NF-kB" FEBS LETTER, vol. 402, 1997, pages 85-90, XP002095261 GREAT BRITAIN cited in the application page 89: "Conclusion" see abstract ---	1-5
X	EP 0 553 658 A (SCHAPER & BRUEMMER GMBH) 4 August 1993 see page 3, line -2; claims 1-18 --- -/-	1-5

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

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2 March 1999

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INTERNATIONAL SEARCH REPORT

In tional Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 098 041 A (JOHNSON EDWARD STEWART ;HYLANDS PETER JOHN (GB); HYLANDS DEBORAH M) 11 January 1984 see claims 1,15 ----	1-5
X	WO 92 11857 A (RHODES TECHNOLOGY LTD) 23 July 1992 see page 1, line 9-15 ----	1-5
X	WO 94 06800 A (MADECA ;MENGAL PHILIPPE (FR); MONPON BERNARD (FR)) 31 March 1994 see page 2, line 1-4; claims 1-15 ----	1-5
A	BALDWIN ET AL: "THE NF-KB AND IκB PROTEINS: New Discoveries and Insights" ANNU REV IMMUNOL, vol. 14, 1996, pages 649-683, XP002095262 USA cited in the application see page 672, last paragraph -----	1-5

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB 98/02108

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